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First report of *Leptographium pini-densiflorae* causing sapstain on *Pinus densiflora* in Korea

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Abstract —Morphological observation and phylogenetic analysis revealed that the dominant sapstain fungus isolated during a recent survey of sapstain of Japanese red pine logs in Korea represents *Leptographium pini-densiflorae*, a new record for Korea. A detailed description of *L. pini-densiflorae* accompanies illustrations of cultural and microscopical characters.

Key words — ascomycetes, blue-stain fungi, taxonomy

Introduction

Species of *Leptographium* Lagerb. & Melin are well known as agents of sapstain in conifers and have been reported worldwide (Seifert et al. 2013). Some *Leptographium* spp. are known to have a symbiotic relationship with bark beetles such as red turpentine beetle (*Dendroctonus valens*), pine shoot beetle (*Tomicus piniperda*), and European spruce bark beetle (*Ips typographus*) (Jacobs & Wingfield 2001, Wingfield et al. 1993, Masuya et al. 2003, 2009, Seifert et al. 2013, Solheim et al. 1993, Zhou et al. 2002). Associated insects provide a means of mobility for *Leptographium* spp.

Among the insects associated with *Leptographium* species, some *Dendroctonus* bark beetles cause devastation in Canada and China (Lee et al. 2006, Lu et al. 2009). Lodgepole pine (*Pinus contorta*) forest in British Columbia has suffered from considerable mortality due to the combination of mountain pine beetle (*D. ponderosae*) and *Leptographium longiclavatum* S.W. Lee et al. (Lee et al. 2006). A similar phenomenon happened in northern China, where extensive tree death occurred in forests over large areas. Lu et al. (2009) noted that transportation of unprocessed logs was a dispersal means for the rapid spread of exotic insects and *Leptographium* spp.

Research on sapstain fungi has focused on commercial logs and boards in Korea, where *Grosmannia koreana* (Masuya et al.) Q. Lu et al., *Ophiostoma floccosum* Math.-Käärik, *O. ips* (Rumbold) Nannf., *O. piceae* (Münch) Syd. & P. Syd., *O. piliferum* (Fr.) Syd. & P. Syd., and *O. quercus* (Georgev.) Nannf. have been reported (Kim et al. 2005, 2007, 2010, 2011). Among the ophiostomatoid fungi, *Grosmannia koreana* [= *Leptographium koreanum* J.J. Kim & G.H. Kim,] was the most dominant sapstain fungal species isolated from conifer logs and wood products in Korea (Masuya et al. 2005, Kim et al. 2005, 2007). However, during recent surveys of log yards, another *Leptographium* sp. was found dominant in Japanese red pine (*Pinus densiflora* Siebold & Zucc). Morphological and phylogenetic analyses revealed that the isolates represent *Leptographium pini-densiflorae*, which has not been reported previously in Korea. Detailed description and figures for this species are provided.

Materials & methods

Morphological examination

Leptographium isolates were collected from the sapwood of Japanese red pine, at Mt. Chiak and the Bonghwa log yard in Korea. The fungus was isolated by inoculating 2% malt extract agar (MEA, malt extract 20 g, agar 15 g, distilled water 1000 ml) containing cycloheximide with a small piece of sapwood (Masuya et al. 2000). The pure isolate was subcultured on 2% MEA and incubated at 20°C for 14 days for morphological investigation. For microscopic characteristics, fungal structures were mounted in 85% lactic acid and observed at ≤1000× using an Olympus BX51 light microscope. Fifty measurements were made for each character. Isolates were deposited in the Korean Agricultural Culture Collection, National Academy of Agricultural Science, Jeonju-si, Korea (KACC) and the Korea University Culture Collections, Korea University, Seoul, South Korea (KUC).

Phylogenetic analysis

Genomic DNA was extracted according to Jang et al. (2013a). PCR reactions were performed for the: [1] internal transcribed spacer and part of the large subunit (ITS2-28S), [2] translation elongation factor-1α (TEF), and [3] β-tubulin (TUB) region using the primers [1] ITS3 / LR3 (White et al. 1990), [2] EF1F / EF2R (Jacobs et al. 2004),

Table 1. Fungal species and GenBank accession numbers of sequences used in this study.

Species	Isolate numbers[a]	GenBank Accession No.		
		ITS2-28S	TUB	TEF
Grosmannia serpens	CMW 304, CBS 141.36	JN135314	JN135334	JN135307
Leptographium bhutanense	CMW 18650	EU650186	EU650190	EU650194
	CMW 18649	EU650187	EU650191	EU650195
L. gracile	CMW 12316	HQ406842	HQ406890	HQ406866
	CMW 12398	HQ406840	HQ406888	HQ406864
L. latens	CMW 12319	HQ406844	HQ406892	HQ406868
	CMW 12438	HQ406845	HQ406893	HQ406869
L. manifestum	CMW 12461	HQ406838	HQ406886	HQ406862
	CMW 12436	HQ406839	HQ406887	HQ406863
L. pini-densiflorae	CMW 25611	EU785398	EU785371	EU785427
	CMW 25600	EU785397	EU785370	EU785426
	CMW 5158	AY707200	KM491367	KM491467
	CMW 5157	AY707199	KM491366	KM491475
	KACC 47825	KP264113	KM058085	KP264112
L. procerum	MUCL46357	EU502795	EU502806	EU502821
	MUCL47244	EU502796	EU502807	EU502822
L. profanum	CMW 10550	DQ354943	KM491375	KM491484
	CMW 10552	DQ354944	KM491376	KM491485
L. sibiricum	CMW 4482	KM491425	KM491379	KM491488
	CMW 4481	KM491424	KM491378	KM491487
L. sinoprocerum	MUCL 46331	EU296772	EU296778	EU296785
	MUCL 47246	EU296774	EU296780	EU296787
L. terebrantis	ATCC 58098	JF798476	JF798460	JF798471
	CBS 337.7	EU296777	EU296784	EU296791

[a] CMW: Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherland; MUCL: Mycothèque de l'Université catholique de Louvain, Lou-vain-la-Neuve, Belgium; ATCC: American Type Culture Collection, Manassas, Virginia, USA; KACC: Korean Agriculture Culture Collection, Suwon, Korea.

and [3] T10 / BT2b (O'Donnell & Cigelnik 1997, Glass & Donaldson 1995). The PCR conditions followed Jacobs et al. (2004) for ITS2-28S and TEF and Masuya et al. (2000) for TUB. The products were sequenced by Macrogen (Korea) and the obtained sequences were deposited in GenBank (Table 1). The sequences were proofread and compared with reference sequences in GenBank. The phylogenetic tree was inferred according to Jang et al. (2013b). Closely related *Leptographium* sequences (Table 1) were retrieved from GenBank. The sequences were aligned with MAFFT 7.130 (Katoh & Toh 2008) with L-INS-i method and then manually edited using MacClade 4.08 (Maddison & Maddison 2005). The best-fit model was searched by MrModeltest 2.3 (Nylander 2004) under AIC criterion and majority rule consensus tree was generated using MrBayes 3.2.2 (Ronquist et al. 2012). Nodal support was assessed with posterior probabilities. The tree was visualized using FigTree 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Taxonomy

Leptographium pini-densiflorae Masuya & M.J. Wingf.,
Mycoscience 41: 428. 2000. FIG. 1

Conidiophores macronematous, mononematous with rhizoids at the bases, single or occasionally in groups. Stipes erect, olivaceous, cylindrical, 1–6-septate, 43–160(–245) µm (mean = 102.9 µm) long and 3.7–8.7(–11.3) µm (mean = 5.9 µm) wide at base. Conidiogenous apparatus 30-84 µm long (mean = 51 µm) excluding conidial mass, 3–5 (mostly 4) series of branches. Primary branches, 2–4 (mostly 2), without larger central branch, light olivaceous to hyaline, smooth, 8–24.4 × 2.2–6 µm (mean = 12.7 × 3.9 µm). Conidiogenous cells discrete, cylindrical, tapering slightly at the apex, 7–16.6 × 1–2.3 µm (mean = 10.8 × 1.5 µm). Conidia oblong to ellipsoid, sub-truncate at base, widely rounded at apex, 2.7–8.5 × 1.2–3 µm (mean = 4.6 × 1.9 µm).

Colonies with optimal growth at 25°C on 2% MEA, reaching 36 mm in diameter in 6 days. No growth below 5°C or above 35°C. Cycloheximide tolerant with a 65% reduction in growth on 5 g/l cycloheximide after 6 days at 25°C in the dark. Colonies hyaline to light olivaceous. Colony margin smooth. Hyphae submerged in the medium or aerial, light olivaceous to hyaline, verrucose, not constricted at the septa, 1.3–11.6 µm wide.

SPECIMEN EXAMINED: **KOREA**, **Gangwon-do**, Mt. Chiak, 37°24′10″N 128°2′57″E, on log of Japanese red pine, 26 July 2012, Joo-Hyun Hong (KACC47825 = KUC20013).

DISTRIBUTION — China, Japan, Thailand, and Korea.

REMARKS — The characteristics of *Leptographium pini-densiflorae* KUC20013 from Korea are consistent with the data from Masuya et al. (2000). The species was first reported by Masuya et al. (2000) from infected Japanese red pine in association with *Tomicus piniperda* in Japan, and later with *T. minor*, *Cryphalus fulvus*, and *Orthotomicus angulatus*. This species was also reported from *Pinus kesiya* Royle ex Gordon in Thailand and two years later from invasive pine-infesting *Dendroctonus valens* in China (Yamaoka et al. 2007, Lu et al. 2009). After *L. pini-densiflorae* was first discovered in Korea in 2012, it became the major sapstain fungus detected in surveys of Japanese red pine in Korea, and the previously dominant species, *G. koreana,* was no longer found. At present, *L. pini-densiflorae* appears restricted to east and southeast Asia.

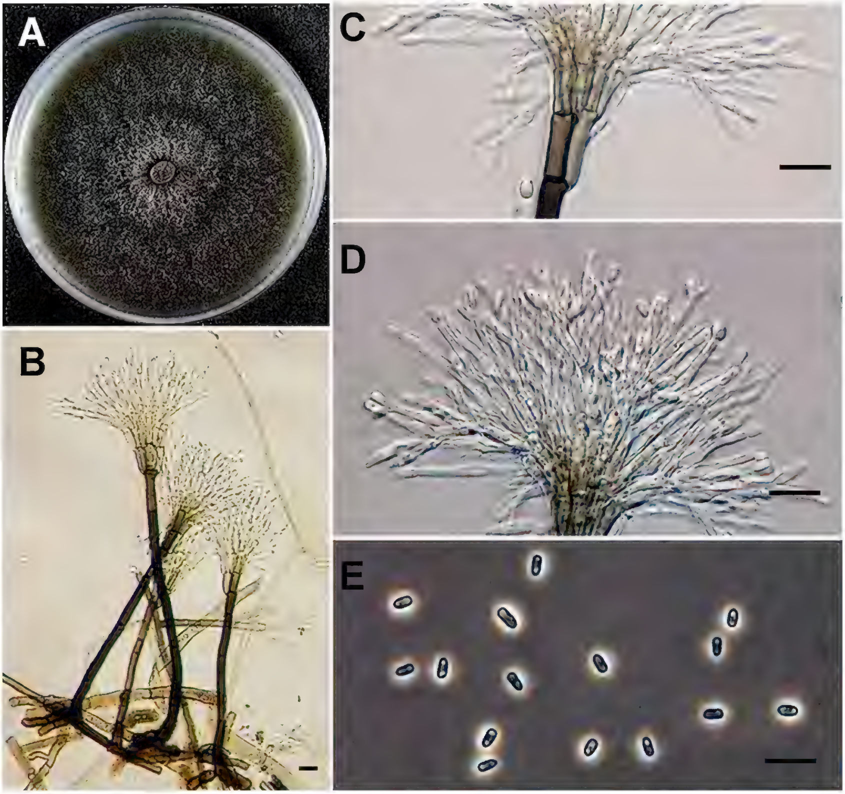

Fig. 1. *Leptographium pini-densiflorae* (KUC20013). A, growth on MEA in 9 cm diam Petri dishes after 14 d at 20°C. B, conidiophore. C, conidiogenous apparatus. D, conidiogenous cells. E, conidia. Scale bar = 10 µm.

Some *Leptographium* species are closely associated with bark beetles, and the species composition of sapstainers can be directly related to the beetle populations. The bark beetles associated with *L. pini-densiflorae* in Korea should be examined.

Phylogeny

Amplification generated sequences of 954 bp from TIS2-28S, 504 bp from TEF, and 442 bp from TUB. The aligned multi-locus dataset comprised 24 isolates and 1365 characters including gaps. The best-fit models for Bayesian phylogenetic analysis were GTR+G for ITS2-28S, HKY+I for TEF, and SYM+G for TUB. *Grosmannia serpens* Goid. (CMW304) was used as outgroup. The 50% majority rule consensus tree using Bayesian analysis is shown in Fig. 2.

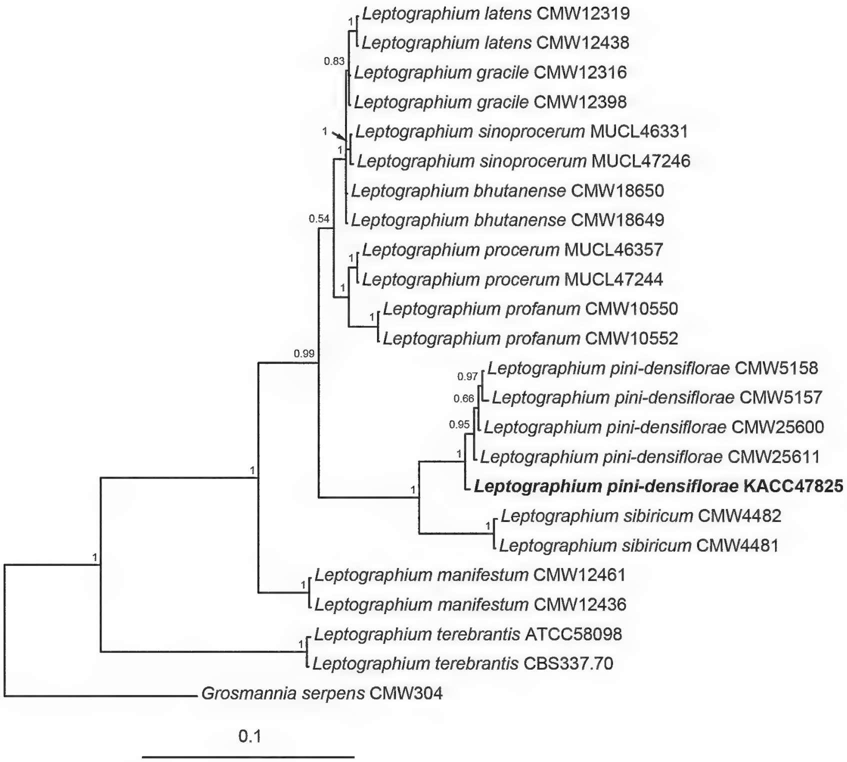
Leptographium latens CMW12319
Leptographium latens CMW12438
Leptographium gracile CMW12316
Leptographium gracile CMW12398
Leptographium sinoprocerum MUCL46331
Leptographium sinoprocerum MUCL47246
Leptographium bhutanense CMW18650
Leptographium bhutanense CMW18649
Leptographium procerum MUCL46357
Leptographium procerum MUCL47244
Leptographium profanum CMW10550
Leptographium profanum CMW10552
Leptographium pini-densiflorae CMW5158
Leptographium pini-densiflorae CMW5157
Leptographium pini-densiflorae CMW25600
Leptographium pini-densiflorae CMW25611
Leptographium pini-densiflorae KACC47825
Leptographium sibiricum CMW4482
Leptographium sibiricum CMW4481
Leptographium manifestum CMW12461
Leptographium manifestum CMW12436
Leptographium terebrantis ATCC58098
Leptographium terebrantis CBS337.70
Grosmannia serpens CMW304
0.83
0.54
0.99
0.97
0.66
0.95
1
0.1

Fig. 2. 50% majority-rule consensus tree of 600 trees resulting from a bayesian analysis of 24 ITS2-28S, TEF, and TUB sequences. Bayesian posterior probabilities ≥50% are shown.

The *Leptographium pini-densiflorae* KUC20013 was monophyletic with the *L. pini-densiflorae* isolates from China (CMW25600 and CMW25611) and Japan (CMW5157 and CMW5158) with high posterior probability (PP = 1); *L. sibiricum* K. Jacobs & M.J. Wingf. was shown as a sister taxon (PP = 1). These two species again clustered with six *Leptographium* spp. (PP = 0.99): *L. bhutanense* X.D. Zhou et al., *L. gracile* Paciura et al., *L. latens* Paciura et al., *L. procerum* (W.B. Kendr.) M.J. Wingf., *L. profanum* K. Jacobs et al., and *L. sinoprocerum* Q. Lu et al., among which four (*L. bhutanense, L. gracile, L. latens, L. sinoprocerum*) are also Asian species (Paciura et al. 2010). Unlike *L. pini-densiflorae*, *L. sibiricum* lacks rhizoids, and although *L. bhutanense, L. gracile*, *L. latens*, and *L. sinoprocerum* produce rhizoids, their colony morphologies, conidiophore lengths, and conidial sizes (Paciura et al. 2010) all differ from *L. pini-densiflorae.*

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